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Determinants in the 5′ Noncoding Region of Poliovirus Sabin 1 RNA That Influence the Attenuation Phenotype

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A number of recombinants between the virulent Mahoney and attenuated Sabin strains of type 1 poliovirus were constructed by using infectious cDNA clones of the two strains. To identify a strong neurovirulence determinant(s) residing in the genome region upstream of nucleotide position 1122, these recombinant viruses were subjected to biological tests, including monkey neurovirulence tests. The results of the monkey neurovirulence tests suggested the important contribution of an adenine residue (Mahoney type) at position 480 to the expression of the neurovirulence phenotype of type 1 poliovirus. This nucleotide, however, had only a minor effect, if any, on viral temperature sensitivity. Monkey neurovirulence tests on the recombinant virus whose genome had a guanine residue (Sabin type) at position 480 and variants generated from this recombinant virus in the central nervous system of monkeys strongly suggested that only one nucleotide change, from adenine to guanine, was not sufficient for full expression of the attenuation phenotype encoded by this genome region. These results suggest that the expression of the attenuation phenotype depends on the highly ordered structure formed in the 5′ noncoding sequence and that the formation of such a structure is possibly influenced by the nucleotide at position 480. Furthermore, in vitro biological tests performed on viruses recovered from the central nervous system of monkeys injected with a temperature-sensitive recombinant virus showing the small-plaque and *d* phenotypes revealed that most of the recovered viruses had even higher temperature sensitivities and that all of the recovered viruses that had acquired the large-plaque phenotype had lost the *d* phenotype to some extent. These results indicate that there may be an unknown selection pressure(s) in the central nervous system and that common determinants might be involved in the expression of the small-plaque and *d* phenotypes.

Poliovirus is a human enterovirus belonging to the *Picornaviridae*. This virus, a nonenveloped particle consisting of a single-stranded RNA genome with positive polarity and 60 copies of each of the four capsid proteins, VP1, VP2, VP3, and VP4, occurs in three stable serotypes, 1, 2, and 3. The virus is known to be the causative agent of poliomyelitis. To control the paralysis caused by poliovirus, attenuated poliovirus strains have been developed and effectively used as oral live vaccines, that is, the Sabin 1 (type 1), Sabin 2 (type 2), and Sabin 3 (type 3) strains. A wealth of data has recently been accumulating on the structure and function of poliovirus. Indeed, the chemical (5) and crystal (4) structures of poliovirus have already been elucidated. The molecular basis of the disease syndrome, however, is not known at present.

The attenuated Sabin 1 strain was derived from the virulent Mahoney strain of type 1 poliovirus by multiple passages through cells of nonhuman origin. These two strains differ strikingly in the potential for causing the disease. Since poliovirus can be transferred to monkeys, in which it also causes paralysis, the neurovirulence phenotype can be determined in experimental animals by monitoring paralysis and the development of histological lesions in the central nervous system after intracerebral (intrathalamic) or intraspinal injection. In addition to the attenuation phenotype, the Sabin 1 strain acquired a number of biological characteristics different from those of the parent Mahoney strain. Some of these characteristics are used as in vitro markers to examine the properties of oral live vaccines. They include the sensitivity of viral multiplication to elevated temperatures (*rct* marker), the sensitivity of viral plaque-forming ability to low concentrations of sodium bicarbonate under an agar overlay (*d* marker), and the size of plaques produced in infected monolayers of primate cells (11).

The differences in biological characteristics between these two strains must be due to the differences in the genome structures resulting from the attenuation process used to obtain the Sabin 1 strain. Comparative sequence studies (14) performed on the genomes of the Mahoney and Sabin 1 strains allowed the identification and mapping of the point mutations in the genome. Several studies (12, 14, 17, 22) revealed 56 nucleotide substitutions among the total 7,441 nucleotides of the genome, not including poly(A) attached at the 3′ terminus. These nucleotide changes are scattered over the entire length of the genome and result in 21 amino acid replacements within the viral polyprotein. Insertions or deletions have not been observed. An important development relating to the identification of the genome region(s) influencing the neurovirulence phenotype as well as in vitro markers was the construction of infectious cDNA clones of both the Mahoney (19, 21) and Sabin 1 (7, 18) strains. The availability of the total nucleotide sequences and infectious cDNA clones of the two strains allowed a molecular genetic approach for investigation of the relationship between the

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structure and function of the viral genome with the use of recombinant DNA technology.

We recently constructed a number of recombinants between the Mahoney and Sabin 1 strains in vitro by using infectious cDNA clones of the two strains (6, 8, 17). Monkey neurovirulence tests on these recombinant viruses (6, 15, 17) revealed that the genome region of nucleotide positions 1 to 1122, including mainly the 5′ noncoding region, harbors a relatively strong determinant(s) influencing the neurovirulence or attenuation phenotype, although determinants weakly influencing the phenotype were spread over several areas of the entire viral genome. This led to speculation that the rate of viral multiplication in the central nervous system might be one of the important factors determining the neurovirulence phenotype (6, 13, 17). In vitro phenotypic marker tests on these recombinant viruses indicated that the *rct* marker might be somewhat correlated with the attenuation phenotype (17) and that the *d* and small-plaque markers were almost completely due to the characteristics of the Sabin 1-derived capsid proteins (6, 17).

Here we investigated the contribution of the genome region upstream of nucleotide position 1122 to the expression of the neurovirulence or attenuation phenotype of type 1 poliovirus. Furthermore, genetic variation of a recombinant virus during replication in the central nervous system of monkeys was investigated. The data indicated the possible contribution of the functional structures formed in the 5′ noncoding region to the expression of the attenuation phenotype. We also discuss the selection pressure(s) existing in the central nervous system of monkeys.

(Preliminary results of this work were presented at the 1988 ICN-UCI International Conference on Virology.)

MATERIALS AND METHODS

Construction of infectious cDNA clones of recombinant viruses. Highly efficient infectious cDNA clones of the Mahoney and Sabin 1 strains were constructed (7) and designated pVM(1)pDS306(T) and pVS(1)IC-0(T), respectively; the vectors contained the replication origin, a promoter, and a coding sequence for the large T antigen of simian virus 40. To construct infectious cDNA clones of recombinant viruses, the cDNA segments of the corresponding nucleotide positions, from 1 to 1813, of the Mahoney and Sabin 1 strains were subcloned into vector plasmid pML2 and designated pEP(M) and pEP(Sab), respectively (9). Allele replacement experiments were carried out on these two plasmids with the restriction enzymes *Kpn*I, *Nco*I, *Fok*I, *Ban*II, and *Aat*II. The resulting plasmids were designated pEP(SM)5a, pEP(SM)6a, pEP(SM)9a, pEP(SM)9b, pEP(SM)10a, and pEP(SM)10b. The shorter *Aat*II fragment of pVS(1)IC-0(T) was replaced by the shorter *Aat*II fragments of these plasmids. As a result, we obtained six new recombinant cDNA clones infectious for mammalian cells and designated pVSM(1)IC-5a(T), pVSM(1)IC-6a(T), pVSM(1)IC-9a(T), pVSM(1)IC-9b(T), pVSM(1)IC-10a(T), and pVSM(1)IC-10b(T), respectively.

Cells and virus stocks. African green monkey kidney (AGMK) cells were maintained in Dulbecco modified Eagle medium supplemented with 5% newborn calf serum. AGMK cells were transfected with 10 μg of the closed circular forms of the recombinant cDNA clones per 60-mm plastic dish by the modified calcium phosphate method described previously (7), and the viruses were recovered from the cells. The viruses recovered from the cells transfected with pVS(1)IC-0(T), pVSM(1)IC-5a(T), pVSM(1)IC-6a(T), pVSM(1)IC-9a(T), pVSM(1)IC-9b(T), pVSM(1)IC-10a(T), pVSM(1)IC-10b(T), and pVM(1)pDS306(T) were designated PV1(Sab) IC-0, PV1(SM)IC-5a, PV1(SM)IC-6a, PV1(SM)IC-9a, PV1 (SM)IC-9b, PV1(SM)IC-10a, PV1(SM)IC-10b, and PV1(M) pDS306, respectively (see Fig. 1), and used as virus stocks. All recombinant viruses were grown at 33.5°C. The titers of virus stocks usually ranged from 1×10^8 to 3×10^8 PFU/ml. The virus stocks of PV1(SM)IC-4a and PV1(SM)IC-4b used in this study were the same as those previously reported by Omata et al. (17).

Virus infection and growth. To measure virus titers, monolayers of AGMK cells in 60-mm plastic dishes were washed twice with Eagle minimum essential medium, covered with 0.5 ml of a virus solution (a dilution of a virus stock), and kept at room temperature for 1 h. After incubation at 35.5°C for 30 min, the cells were washed twice with Eagle minimum essential medium and covered with the same medium containing 1% agarose and 5% newborn calf serum. After 3 to 4 days of incubation at 35.5°C, plaques were visualized by staining cells with crystal violet, and virus titers were calculated on the basis of the numbers of plaques.

To prepare virus solutions for monkey neurovirulence tests and in vitro marker tests, approximately 4×10^7 AGMK cells were infected with each recombinant virus stock at a multiplicity of infection of approximately 10^{-3} and incubated at 33.5°C for about 2 days until all the cells showed cytopathic effects. The infected cell cultures were frozen at appropriate times, and virus solutions were prepared by freezing-thawing three times followed by low-speed centrifugation to remove cell debris. The titers of viruses were measured as described above.

Monkey neurovirulence tests. Before the monkey neurovirulence tests were done, the titers of the viruses were measured again with primary cultured cynomolgus monkey kidney cells. Into the lumbar enlargement of each seronegative cynomolgus monkey 0.1 ml of a virus suspension (10^7 50% tissue culture infective doses per ml) was inoculated. Monkeys showing severe clinical poliomyelitis were sacrificed at the peak of the disease. Other monkeys were sacrificed 17 days after inoculation. A total of 38 sections of the central nervous system were prepared to evaluate the intensity of histological lesions as previously described (17). Lesion scores and spread values were determined by established procedures (2, 23).

Recovery of viruses from the central nervous system. In some cases, we recovered viruses from the central nervous system of monkeys inoculated with viruses. Recombinant viruses were inoculated in the same manner as described above, and the monkeys were sacrificed 7 days after inoculation. The spinal cords and brains of these monkeys were dissected out and cut into five pieces, that is, the thalamus, midbrain and pons, medulla oblongata, cervical cord, and lumbar cord. Each of these pieces was homogenized in Dulbecco modified Eagle minimum essential medium to obtain a 10% (wt/vol) emulsion. AGMK cells (approximately 5×10^6 cells) in 60-mm plastic dishes were infected with 0.2 ml of each emulsion in the same manner as described above and then covered with Eagle minimum essential medium containing 1% agarose and 0.1% bovine serum albumin. After 3 days of incubation at 33.5°C, viruses were prepared by isolating individual plaques with Pasteur pipettes, followed by suspension in 1 ml of Dulbecco modified Eagle minimum essential medium. AGMK cells were infected with these viruses, and virus stocks of individual plaque isolates were obtained.

In vitro phenotypic marker tests. All in vitro phenotypic

marker tests were performed with primary cultured cynomolgus monkey kidney cells. The reproductive capacity at different temperatures (*rct* marker) of viruses was investigated by measuring the virus titers on the cells at a sodium bicarbonate concentration of 0.225% and temperatures of 36, 39, 39.5, and 40°C after incubation for 7 days as described previously (6, 7, 17). For determination of the sizes of plaques produced by the different viruses, cells were infected and cultured under agar overlays at a sodium bicarbonate concentration of 0.225% at 36°C. The diameters of approximately 100 plaques, observed on day 5 postinfection, were determined as described previously (6, 7, 17). Delayed growth (*d* marker) of viruses was investigated by measuring the virus titers on the cells at 36°C and sodium bicarbonate concentrations of 0.225 and 0.03% after incubation for 4 days as described previously (6, 7, 17).

Nucleotide sequence analysis. Approximately 4×10^7 AGMK cells were infected with viruses at 10 to 20 PFU per cell and incubated at 35.5°C for 7 to 12 h, when almost all of the cells showed cytopathic effects. Cytoplasmic RNAs containing poliovirus RNA were prepared from the infected cells by the method of Berk et al. (1) as previously described (9). With this procedure, approximately 100 μg of cytoplasmic RNA containing poliovirus RNA was obtained.

A modified dideoxy method (9) was used for sequencing viral RNAs. The dideoxy method with synthetic DNA primers (20) was used to confirm the modified nucleotide sequences of all recombinant DNAs.

RESULTS

Construction of recombinants between the Mahoney and Sabin 1 strains. The initial monkey neurovirulence tests were performed with intracerebral inoculation to identify the genome region influencing the neurovirulence or attenuation phenotype of type 1 poliovirus (6, 17). Although the genome loci contributing to the expression of the neurovirulence phenotype were shown to be distributed along the entire length of the virus genome, the strong determinant(s) for the expression of the neurovirulence phenotype was shown to reside in the genome region upstream of nucleotide position 1122 (6, 17).

Seven nucleotide differences were observed in the nucleotide sequence upstream of nucleotide position 1122 in the genomes of the Mahoney and Sabin 1 strains. To determine the nucleotide position(s) in this genome region which influences the expression of the neurovirulence phenotype, allele replacement experiments were carried out on the sequence upstream of nucleotide position 1122 (Fig. 1A and B). Various portions of the Mahoney or Sabin 1 sequence existing in the genome of virus PV1(SM)IC-4b or PV1 (SM)IC-4a, respectively, were replaced by the corresponding portions of the Sabin 1 or Mahoney sequence, making use of the restriction enzymes *Kpn*I, *Nco*I, *Fok*I, *Ban*II, and *Aat*II as described in Materials and Methods. As a result, we constructed six different recombinant viruses, PV1(SM)IC-5a, PV1(SM)IC-6a, PV1(SM)IC-9a, PV1(SM)IC-9b, PV1 (SM)IC-10a, and PV1(SM)IC-10b. The genome structures of these recombinant viruses are shown in Fig. 1A and B. Nucleotide sequence analyses performed on RNA genomes of these recombinant viruses as well as their cDNAs revealed that all of the recombinant viruses had the expected modified nucleotide sequences.

Monkey neurovirulence tests with recombinant viruses. To compare the neurovirulence levels of the recombinant viruses, we determined the experimental conditions under

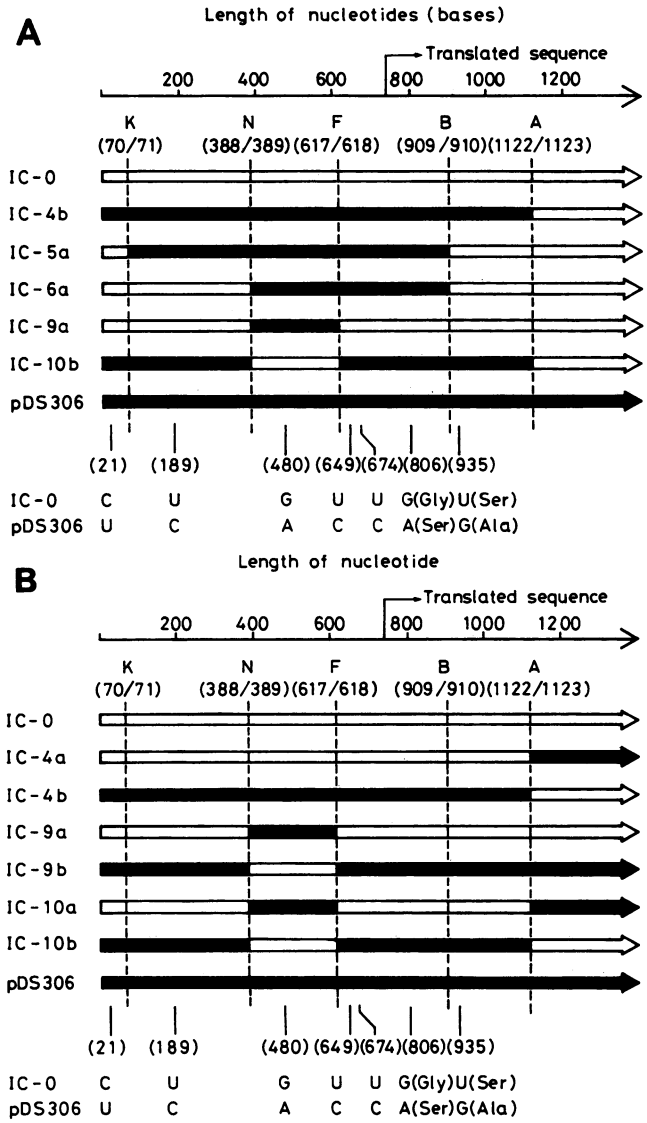

FIG. 1. Genome structures of recombinant type 1 polioviruses. The expected genome structures of the recombinant viruses are shown as a combination of the Sabin 1 (▭) and Mahoney (▬) sequences. K, N, F, B, and A denote cleavage sites of restriction enzymes *Kpn*I, *Nco*I, *Fok*I, *Ban*II, and *Aat*II, respectively. The numbers in parentheses are the nucleotide positions from the 5′ end of the genome. The length of the genome of the type 1 poliovirus is shown at the top of each figure in bases from the 5′ terminus. Nucleotide and amino acid differences between the Mahoney and Sabin 1 strains are shown at the bottom of each figure. VPg, a small protein covalently attached to the 5′ end of the genome, is omitted from the figure. The nomenclature for virus strains, indicated on the left side of the figure, was modified in that PV1(Sab), PV1(SM), and PV1(M) were omitted.

which virus PV1(SM)IC-4b showed the greatest extent of lesion spread in the central nervous system from the injection site on the lumbar enlargement. Spinal injection of virus PV1(SM)IC-4b at a 50% tissue culture infective dose of 10^6 viruses per monkey was found to give the highest lesion score (approximately 2.0), whereas the Sabin 1 strain gave a lesion score of approximately 0.9 (Fig. 2). Thus, we adopted these conditions for the monkey neurovirulence tests in this study. Since these experimental conditions must result in the death of all monkeys injected with the virulent Mahoney

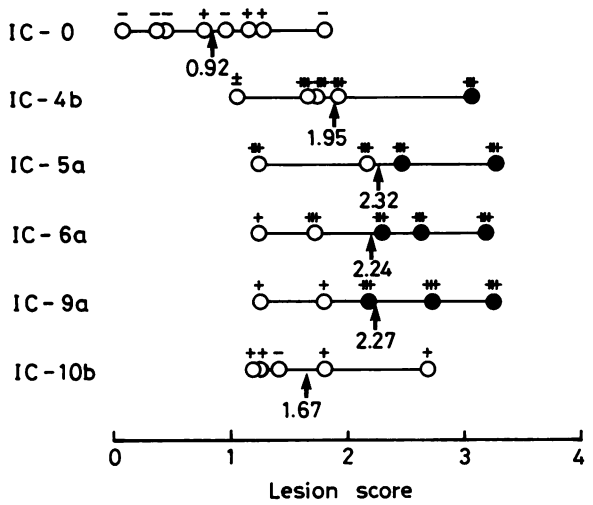

FIG. 2. Results of monkey neurovirulence tests with recombinant viruses. The lesion scores for individual monkeys injected with recombinant viruses or the Sabin 1 virus are plotted with the intensities of paralysis. Closed circles are for monkeys that died during the course of the test. The average lesion score for each virus is indicated by an arrow. As to the nomenclature, PV1(Sab) and PV1(SM) were omitted, as in Fig. 1.

strain, we used the PV1(SM)IC-4b and Sabin 1 viruses as controls.

Five different recombinant viruses and the Sabin 1 strain (Fig. 1A) were tested for neurovirulence by intraspinal injection into cynomolgus monkeys. The results of intraspinal neurovirulence tests with these viruses are shown in Fig. 2. It should be noted that the lesion scores do not show first-degree linearity. Therefore, the intensity of neurovirulence cannot be simply represented by the average lesion score but should be judged by taking the clinical symptoms, including paralysis and death, into consideration in addition to the histological lesion score (Fig. 2). From this viewpoint, viruses PV1(SM)IC-5a, PV1(SM)IC-6a, and PV1(SM)IC-9a were as neurovirulent as or slightly more neurovirulent than PV1(SM)IC-4b. Considering the nucleotide differences between the genomes of the Mahoney and Sabin 1 strains, virus PV1(SM)IC-9a is a one-point mutant of the Sabin 1 strain, with regard to nucleotide position 480 (Fig. 1A). Thus, it appeared that an adenine residue at nucleotide position 480 greatly influenced the neurovirulence phenotype expressed by the 5'-proximal 1,122 nucleotides. The data also appeared to confirm our previous assumption that the 5' noncoding region plays a crucial role in the expression of neurovirulence.

Since the existence of an adenine residue at nucleotide position 480 appears to be enough for the neurovirulence phenotype expressed by the genome region of the 5'-proximal 1,122 nucleotides, it is possible that only one nucleotide exchange (adenine ↔ guanine), at nucleotide position 480, functions like a switch determining which of the two phenotypes, the neurovirulence or attenuation phenotype, is expressed, with regard to the gene function of the 5' noncoding sequence. If this assumption is correct, virus PV1(SM)IC-10b must have the attenuation phenotype of the Sabin 1 virus, since the genome of the recombinant virus has a guanine residue at position 480 and since the sequence downstream of position 1123 is derived from the Sabin 1 virus (Fig. 1A). No monkey died during the course of the neurovirulence test with virus PV1(SM)IC-10b, as during that with the Sabin 1 virus, although virus PV1(SM)IC-10b did not show the attenuation phenotype of the Sabin 1 virus, as seen by comparison of the incidences of paralysis and lesion scores between the PV1(SM)IC-10b and Sabin 1 viruses (Fig. 2). These parameters clearly indicated that virus PV1(SM)IC-10b had a stronger neurovirulence phenotype than did the Sabin 1 virus. These results may suggest that only one nucleotide change (adenine ↔ guanine), at nucleotide position 480, is not sufficient for expression of the attenuation phenotype, probably because expression of the attenuation phenotype depends not on the primary structure in the vicinity of position 480 but on the highly ordered structures formed in the 5' noncoding sequence. Alternatively, the genome structure of PV1(SM)IC-10b may be unstable during replication in the central nervous system of monkeys and variants derived from this virus may cause the development of stronger lesions than expected.

Temperature sensitivity. The temperature sensitivity of virus multiplication has been suggested to exhibit some correlation with the attenuation phenotype (17). Accordingly, *rct* marker tests were carried out on the recombinant viruses whose genome structures are shown in Fig. 1 bottom to determine the effect of the mutation at position 480 on the in vitro marker. Three viruses, PV1(SM)IC-4a, PV1(SM)IC-9a, and PV1(SM)IC-10a, have the Mahoney genome in the region downstream of nucleotide position 1123. The results of the *rct* marker tests are shown in Table 1. The data for recombinant viruses PV1(SM)IC-4a and PV1(SM)IC-4b, when compared with those for parental viruses PV1(M)pDS306 and PV1(Sab)IC-0, respectively, confirmed our previous results (17) showing that the 5'-proximal segment of 1,122 nucleotides includes mutations that influence viral temperature sensitivity.

The temperature sensitivity of virus PV1(SM)IC-4a was slightly higher than those of viruses PV1(SM)IC-9b and PV1(SM)IC-10a. The data suggest that the nucleotide at position 480 is a weak determinant of the *rct* phenotype and that a mutation site other than position 480 is also a determinant(s) of the phenotype. This was also true when the

TABLE 1. Reproductive capacity of viruses at different temperatures

Virus	Log_{10} PFU/ml at temp (°C):				Difference in log_{10} PFU/ml between temp (°C):		
	36	39	39.5	40	36 and 39	36 and 39.5	36 and 40
PV1(Sab)IC-0	8.64	2.87	<0.39		5.77	>8.25	
PV1(SM)IC-4a	7.99	7.08	4.47	2.88	0.91	3.52	5.11
PV1(SM)IC-4b	8.31	6.92	3.05	<0.39	1.39	5.26	>7.92
PV1(SM)IC-9a	8.85	5.44	<0.39	<0.39	3.41	>8.46	>8.46
PV1(SM)IC-9b	8.74		7.70	7.70		1.04	1.04
PV1(SM)IC-10a	8.74	8.44	7.21	5.21	0.30	1.53	3.62
PV1(SM)IC-10b	8.30	5.21	0.70	<0.39	3.09	7.60	>7.91
PV1(M)pDS306	8.86		8.02	7.68		0.84	1.18

result for virus PV1(SM)IC-4b was compared with those for viruses PV1(SM)IC-9a and PV1(SM)IC-10b. On the contrary, a comparison of the results for viruses PV1(SM)IC-9a and PV1(SM)IC-9b with those for parental viruses PV1 (Sab)IC-0 and PV1(M)pDS306, respectively, suggested that the mutation at position 480 had almost no effect on the temperature sensitivity of the viruses. These results indicate that the nucleotide at position 480 is a very weak determinant, if at all, for viral temperature sensitivity. It is very possible, however, that the expression of temperature sensitivity is also due to the functional highly ordered structures in the 5′ noncoding sequence and not to the primary sequence.

Genetic variation of poliovirus in the central nervous system of monkeys. As mentioned above, recombinant virus PV1(SM)IC-10b was much more neurovirulent than expected. There are two possible explanations for this phenomenon. One is that virus PV1(SM)IC-10b itself is more neurovirulent than the Sabin 1 virus. The other is that the unexpectedly high lesion score in the case of monkeys injected with PV1(SM)IC-10b was due to variant viruses generated from virus PV1(SM)IC-10b during replication in the central nervous system.

To determine the stability of the genotype and the phenotype of virus PV1(SM)IC-10b in the central nervous system, the virus was injected intraspinally into two cynomolgus monkeys. Seven days after injection, when the monkeys showed paralysis, viruses were recovered from their central nervous systems as described in Materials and Methods. Viruses could only be recovered from the lumbar cords of the monkeys. The extract of the lumbar cords thus obtained was used as an inoculum for isolating single plaques; 38 plaque-purified isolates were obtained. Nucleotide sequence analysis of the 5′ noncoding regions of the genomes of 18 randomly selected isolates from the extract from the first monkey (viruses 1 through 18 in Table 2) and in vitro phenotypic marker testing of all 38 isolates, 18 from the first monkey (Table 2, experiment 1) and 20 from the second monkey (Table 2, experiment 2), were performed. Nucleotide sequence analysis revealed that the sequences of the 5′ noncoding regions, incuding nucleotide position 480, were found to be unchanged in the genomes of the recovered viruses tested (data not shown). Thus, the 5′ noncoding sequence of PV1(SM)IC-10b seems to be fairly stable during replication in the central nervous system. However, many variants were detected in in vitro biological tests, including *rct*, *d*, and plaque size marker tests. The results of in vitro biological tests are shown in Table 2; the variants could be classified into three groups based on their *rct* phenotype.

Viruses in the first group showed higher temperature sensitivity than did the Sabin 1 virus. Since these viruses showed almost the same in vitro phenotypes as did those of the virus mixture obtained by expansion from approximately 100 PFU of the 10% emulsion of viruses prepared from lumbar cords as described in Materials and Methods (Table 2), the first group appears to represent the major population of the recovered viruses. The second group includes the viruses showing temperature sensitivity similar to that of the Sabin 1 virus. Their temperature sensitivity was also higher than that of the parent virus, PV1(SM)IC-10b. The third group includes the viruses showing temperature sensitivity similar to that of the parent virus. Therefore, virus PV1(SM)IC-10b itself may be included in the viruses of this group.

It is of interest that no recovered virus showed a temperature sensitivity lower than that of the parent virus,

TABLE 2. In vitro markers of recovered viruses and their parental viruses

Expt	Virus	Result for:		Plaque size (mm) (mean ± SD)[c]
		rct[a]	*d*[b]	
1	PV1(Sab)IC-0	5.34	4.28	5.7 ± 2.8
	PV1(SM)IC-10b	3.24	4.34	4.6 ± 1.9
	PV1(M)pDS306	0.16	0.76	11.1 ± 3.2
	Recovered viruses (virus mixture)	>7.44	>5.29	5.0 ± 2.4
	Group 1			
	4	>7.33	>5.24	5.0 ± 2.2
	6	>6.90	>4.75	5.0 ± 2.1
	7	>6.82	>4.72	5.1 ± 2.5
	8	>7.24	>5.15	5.4 ± 2.6
	10	6.71	>5.37	5.3 ± 2.4
	11	>7.26	>5.15	5.4 ± 2.3
	12	>7.31	>5.15	5.1 ± 1.8
	13	>7.42	4.17	5.3 ± 2.4
	14	>7.37	>5.21	5.8 ± 2.5
	15	>7.05	>4.71	5.4 ± 2.6
	16	>7.18	>4.54	5.6 ± 2.1
	17	>6.78	4.62	6.1 ± 2.4
	18	>6.59	>4.51	5.4 ± 2.4
	Group 2			
	2	6.30	4.00	5.4 ± 2.4
	3	5.62	1.30	10.0 ± 2.9
	9	5.98	3.97	5.3 ± 2.2
	Group 3			
	1	3.57	1.29	8.8 ± 4.3
	5	4.30	2.49	10.0 ± 2.9
2	PV1(Sab)IC-0	4.82	4.28	5.0 ± 2.4
	PV1(SM)IC-10b	2.46	4.66	4.3 ± 2.2
	PV1(M)pDS306	0.28	0.78	12.7 ± 2.3
	Recovered viruses			
	Group 1			
	24	6.18	3.68	5.8 ± 2.3
	25	5.86	3.55	4.8 ± 2.2
	28	6.14	4.36	2.9 ± 1.0
	Group 2			
	21	4.11	4.76	3.5 ± 1.4
	26	4.37	3.89	4.7 ± 3.0
	29	5.55	4.50	3.3 ± 1.4
	30	5.32	4.01	3.2 ± 1.3
	33	4.61	3.75	4.8 ± 2.1
	34	3.91	4.21	3.7 ± 1.5
	37	5.26	2.66	3.8 ± 3.1
	Group 3			
	19	1.97	3.78	5.4 ± 1.8
	20	2.35	4.22	6.0 ± 2.1
	22	2.55	3.85	4.8 ± 2.3
	23	2.74	3.95	5.5 ± 2.4
	27	2.79	3.13	6.2 ± 2.6
	31	2.75	3.43	5.6 ± 2.4
	32	2.77	3.53	6.2 ± 3.0
	35	2.94	3.19	4.4 ± 1.9
	36	2.71	3.57	3.9 ± 1.5
	38	2.94	3.67	5.3 ± 3.3

[a] Values are the logarithmic differences between virus titers obtained at 36 and 39°C [except for PV1(M)pDS306, for which the values obtained at 36 and 39.5°C are given].

[b] Values are the logarithmic differences between virus titers obtained at sodium bicarbinate concentrations of 0.225 and 0.03%.

[c] Plaque size displayed on day 5 of growth.

TABLE 3. Reproductive capacity of passaged viruses at different temperatures

Virus	Passage no.	Log_{10} PFU/ml at 36°C	Difference in log_{10} PFU/ml between temp (°C):	
			36 and 36	36 and 39.5
PV1(Sab)IC-0	0	8.94	5.13	>8.55
	3	7.60	4.38	6.79
	5	8.02	1.81	3.72
PV1(SM)IC-10b	0	8.50	3.51	6.96
	3	8.23	3.05	5.47
	5	7.92	2.50	4.48
Recovered virus mixture		8.76	6.19	>8.37

PV1(SM)IC-10b, and most of them showed a temperature sensitivity even higher than that of the parent virus. This observation is not compatible with the view that a virus must lose its temperature sensitivity during replication at elevated temperatures. To determine whether this phenomenon is limited to viral replication in the central nervous system, the *rct* marker test was performed on PV1(SM)IC-10b preparations passaged in primary cultured cynomolgus monkey cells up to five times at 37.5°C (Table 3). Virus PV1(SM)IC-10b appeared to lose its temperature sensitivity during passaging in the in vitro culture system at 37.5°C, although the phenotype was much more stable than that of the Sabin 1 virus (Table 3). These results suggested that an unknown selection pressure is involved in the generation of the variant viruses in the central nervous system and that virus PV1(SM)IC-10b has an exceptionally stable *rct* phenotype in vitro. It should be noted that temperature-sensitive virus PV1(SM)IC-9a had a very stable *rct* phenotype during replication in the central nervous system (data not shown).

In any event, it was possible that variants more neurovirulent than virus PV1(SM)IC-10b itself existed in the recovered viruses. Therefore, we selected two viruses and then examined their neurovirulence phenotypes. One, virus 1, belonged to the third group, with the weakest temperature sensitivity among the recovered viruses, and had *d* and plaque size phenotypes similar to those of the virulent Mahoney strain. The other, virus 2, had properties typical of viruses belonging to the second group. The results of the monkey neurovirulence tests are shown in Fig. 3. Viruses 1 and 2 showed attenuation phenotypes stronger than that of the parental virus, PV1(Sab)IC-10b, and neurovirulence phenotypes similar to or slightly weaker than that of the Sabin 1 virus (Fig. 3). These data suggested that every variant derived from virus PV1(SM)IC-10b was less neurovirulent than the parent virus, PV1(SM)IC-10b. It is unlikely that recovered viruses not tested for neurovirulence in this study included viruses more neurovirulent than PV1(SM)IC-10b because of the absence of viruses whose multiplication rates were much higher than that of virus 1 in the preparation of the recovered viruses. Therefore, we conclude that the neurovirulence displayed by monkeys injected with virus PV1(SM)IC-10b was due to the neurovirulence of PV1 (SM)IC-10b itself and not to that of variants derived from PV1(SM)IC-10b, although not all of the variants were tested for monkey neurovirulence. These results support the notion that the expression mechanism(s) for the attenuation phenotype is complicated and probably involves highly ordered functional structures formed in the 5′ noncoding sequence. The formation of such structures may be influenced to some extent by the residue at nucleotide position 480. A similar structural change may result from a nucleotide substitution at position 472 (uracil ↔ cytosine) of the type 3 poliovirus genome, although this substitution apparently provides clearer and more consistent results (3) than those described here. The reason for the discrepancy between the results for the type 1 and type 3 polioviruses is not clear at present.

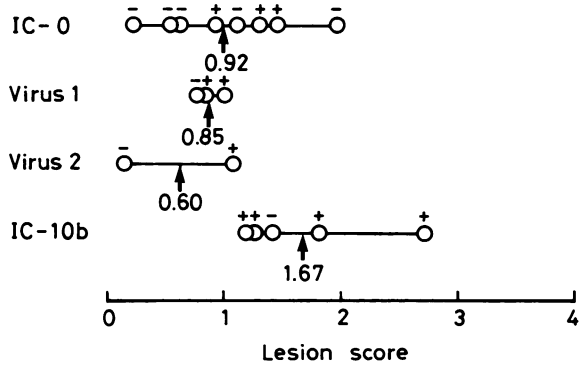

FIG. 3. Results of monkey neurovirulence tests with recombinant viruses and viruses recovered from the central nervous systems of monkeys. Viruses 1 and 2 were prepared by isolating plaques of viruses recovered from the central nervous systems of monkeys as described in Materials and Methods. The lesion scores and the intensities of paralysis for individual monkeys are as in Fig. 2.

DISCUSSION

We constructed recombinants between the virulent Mahoney and attenuated Sabin 1 strains of poliovirus; the genome region upstream of nucleotide position 1122, which is considered to carry a strong determinant(s) of viral neurovirulence was changed (17). Biological tests, including monkey neurovirulence tests, were performed on these recombinant viruses to elucidate the effects of mutations observed in the genome region on the neurovirulence phenotype. The results suggest that highly ordered structures formed in the 5′ noncoding sequence are involved in the expression of the phneotype and that nucleotide position 480 plays an important role in the formation of such highly ordered structures. Thus, elucidation of the highly ordered structures of the 5′ noncoding sequence of the genome appears to be essential for understanding the expression mechanism for the neurovirulence or attenuation phenotype.

Only one nucleotide substitution, at position 480, was found to provide a totally different secondary structure of the 5′ noncoding sequence upon analysis by computer programs which predict the secondary structure with the lowest free energy (data not shown). A similar result was obtained by Evans et al. (3) in the case of the type 3 poliovirus. However, these results are not likely because highly ordered structures essential for viral replication should remain in every genome of viable polioviruses. We are currently attempting to elucidate the secondary structures of the 5′ noncoding sequences of poliovirus genomes by using a new computer program that was developed to predict the number and probability of possible alternate secondary structures (24).

Viruses recovered from the central nervous system of monkeys injected with virus PV1(SM)IC-10b showed interesting biological characteristics. First, most of the recovered viruses were variants that acquired temperature sensitivities

higher than that of parental virus PV1(SM)IC-10b. The mechanism underlying this phenomenon is obscure at present. It is possible, however, that the revertants that had a mutation(s) providing lower temperature sensitivity were selectively eliminated because the mutation(s) resulted in a more serious deficiency in viral replication in the central nervous system. Alternatively, virus PV1(SM)IC-10b and the revertants with lower temperature sensitivities may have had strong and rapid killing effects on the nerve cells in the system, resulting in a reduction in the final yield of infectious particles. It is also possible that revertants with higher temperature sensitivities had weak and slow killing effects, resulting in a predominant population of temperature-sensitive viruses in the central nervous system. Indeed, it has been suggested that the cell killing function of poliovirus is not always correlated with virus production (16). In any event, an unknown selection pressure may be involved in this phenomenon. Second, three variants derived from PV1(SM)IC-10b, viruses 1, 3, and 5, showed a large-plaque phenotype like that of the virulent Mahoney strain (Table 2). The results of *d* marker tests with the three variants revealed that the *d* phenotype was also shifted toward the property of the Mahoney strain (Table 2). These results supported our previous assumption that common determinants may be involved in the expression of the *d* and plaque size phenotypes (6, 17). Identification of the determinants for these two phenotypes is currently in progress with these variant viruses.

Besides the strong neurovirulence determinant investigated in this study, several weak determinants influencing the neurovirulence or attenuation phenotype of type 1 poliovirus appear to be scattered in the genome downstream of nucleotide position 1123 (6, 17). However, it may be impossible to identify these weak determinants in the genome as long as experiments involve monkeys because of the difficulty in obtaining statistically reliable results. Therefore, it is desirable for future studies on neurovirulence to develop a new animal test model. A transgenic mouse model involving a human or monkey gene encoding a cellular receptor for poliovirus infection may provide a means of testing the phenotype, since a recent study (10) revealed that mouse neurovirulence caused by mouse-adapted poliovirus strains seemed to reflect the characteristics of the virus for humans. We are currently attempting to establish a transgenic mouse model for testing the neurovirulence of polioviruses.

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